



Autoantigens Underlying IgG4 Related Disease

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AUTOANTIGENS IN IGG4 RELATED DISEASE

IMAD AWAN

A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

Boston, Massachusetts.

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AUTOANTIGENS IN IGG4 RELATED DISEASE

Abstract

Immunoglobulin G4-related disease (IgG4-RD) is a newly emerging immune-mediated condition that can affect over a dozen organs. The systemic nature of this disease allows it to exhibit a plethora of diverse disorders, including malignancies, infections and also inflammatory diseases. This diversity is evident in the highly heterogeneous patient population, from pancreatic cancer patients to patients with Mikulicz's disease. Such disorders were often diagnosed as isolated, single-organ diseases until the early 21st century, when IgG4-RD was identified by a set of histopathological characteristics seen across patient biopsies. The three hallmark pathological features —lymphoplasmacytic infiltration, storiform fibrosis and obliterative phlebitis— are vital today for a definitive diagnosis. The ability to accurately diagnose IgG4-RD has transformed the outlook for patients, as it is treatable, with patients generally responsive to immunosuppressive therapies.

Over the past decade, we have gained great insight into the widespread immune dysregulation that takes place in IgG4-RD, such as elevated levels of IgG4 antibodies and the presence of characteristic CD4⁺ cytotoxic T lymphocytes in tissue lesions. However, the etiology of the disease is still unknown and it is unclear whether the IgG4 autoantibodies are drivers of the disease or a mere consequence of a robust inflammatory response.

My thesis aims to address these critical questions by identifying the target antigen(s) of these autoantibodies. Essentially, this would provide insight into the pathogenesis in IgG4-RD and help elucidate possible drivers of the dysregulated immune response.

In this paper, I discuss the twenty likely antigenic targets of IgG4 autoantibodies in patient serum, which were identified elegantly by the parallel analysis of translated ORF's (PLATO). These antigens were then synthesized via large-scale transfection of 293F cells. Lastly, the specificity of patient antibodies against these recombinant proteins was then evaluated using ELISA, testing sera from over 100 IgG4-RD patients.

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I would like to thank Dr. Shiv Pillai of the Immunology program at Harvard Medical School who served as my mentor, teacher and thesis advisor. His guidance and support played an immense role in my success over the past two years, and he continues to inspire me today.

I would also like to thank Dr. Cory Perugino of the Rheumatology program at Massachusetts General Hospital. This thesis would not have been possible without his mentorship, support and extensive contribution to this project.

I am also grateful to the other members of the Pillai Lab, notably Vinay Mahajan, Ian Rosenberg, Hamid Mattoo and Xin Kai for helping me master various lab techniques in this project. In addition, I would like to acknowledge the Elledge lab for their key role in PLATO and Grant Weaver at the Ragon Institute of MGH, MIT and Harvard for his guidance in protein production and purification.

Finally, I must express my profound gratitude to my parents and sister who provided the unwavering support and motivation that I need to thrive in all my years of study, and in completing my thesis and achieving a Masters of Medical Sciences in Immunology from Harvard Medical School.

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Chapter One

Background

Immunoglobulin G4-related disease (IgG4-RD) is an emerging systemic immune-mediated condition that exhibits a wide variety of disorders. It is a multi-organ condition that can affect up to a dozen organs to resemble malignancies, infectious and inflammatory diseases, such as pancreatic cancer, autoimmune pancreatitis and Mikulicz's disease. These disorders were often diagnosed as isolated, single-organ diseases until the early 21st century, when IgG4-RD was identified by a number of histopathological characteristics seen across patient biopsies.² The three hallmark pathological features —lymphoplasmacytic infiltration, storiform fibrosis and obliterative phlebitis— remain key today for a definitive diagnosis. This has proved crucial in transforming the outlook for patients, as IgG4-RD is treatable, with patients generally responsive to immunosuppressive therapies.

Epidemiology

Over the past decade, we have gained great insight into the clinical and pathophysiological manifestations of IgG4-RD. However, the epidemiological data continues to remain scarce due to the relatively young age of the disease as well as the difficulty in recognition. Unlike many classic autoimmune disorders, IgG4-RD has a predilection for men, with 3.5 times as many male patients than female. Although there are no known familial cases of IgG4-RD, genetic studies have shown a bias in major histocompatibility complex class II in men of Asian descent³. Collecting accurate epidemiological data will only be possible once awareness of IgG4-RD and its diagnosis increases amongst practitioners.

Clinical Manifestations & Organ Involvement

IgG4-RD patients present with symptoms and organ dysfunction for extended periods of time prior to diagnosis. Common symptoms include fatigue, musculoskeletal symptoms or significant weight loss (5-10 kilograms)³. Disease progression is rarely continuous, with patients frequently showing spontaneous, but temporary improvements. IgG4-RD is suspected either when disease recurs in a particular organ or if additional organs are affected. Discussed below are a few of the extensive organs involved in IgG4-RD.

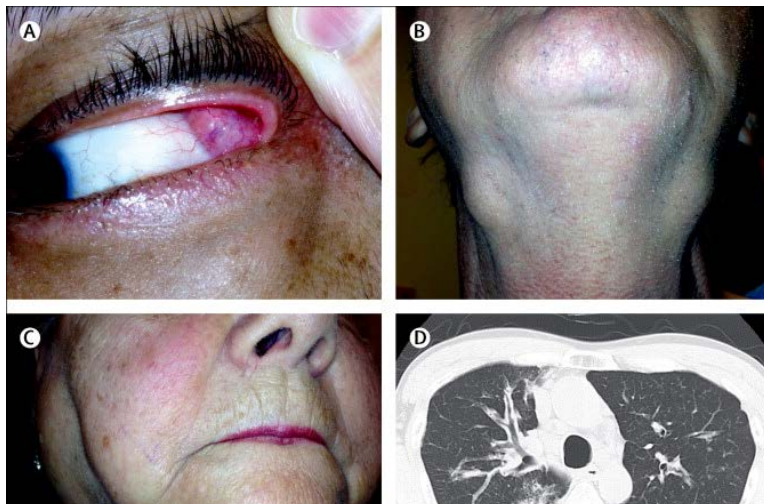


Figure 1. Clinical Manifestations in IgG4-RD patients.

A) Dacryoadenitis, caused by lacrimal gland enlargement. B) Submandibular gland enlargement. C) Parotid enlargement. D) Thickening of bronchovascular bundle in right lung. *Picture Credit: Kamisawa et. al²*

Head and Neck

IgG4-RD patients encompass a wide variety of clinical manifestations in the head and neck regions. In fact, multiple diseases, such as Kuttner's tumor and Mikulicz disease, are now recognized as classic IgG4-related conditions. Identified over 100 years ago, Mikulicz disease entails

enlargement of the parotid, submandibular and lacrimal glands— all common findings in IgG4-RD.

Patients further present with a wide range of orbital and periorbital lesions, ranging from tumefactive lesions to enlarged lacrimal-glands (dacryoadenitis). These lesions are extensive and

may infiltrate along the trigeminal nerve or into the cavernous sinus, and are thus misidentified as malignancies.

Prominent allergic features are also evident in a number of patients, specifically in the ears, nose and throat. These patients tend to have a history of allergies long before an IgG4-RD diagnosis, and sometimes have ten times the upper limit of IgE levels in serum³. Further, diffuse inflammation is seen in the pharynx, hypopharynx and trachea. Mass lesions in the sinuses and destructive lesions in the middle ear and facial bones have also been reported.

Chest

The lungs exhibit the greatest diversity of clinical presentations, which can be classified into four distinct syndromes: inflammatory pseudotumor, central airway disease, localized/diffuse interstitial pneumonia and pleuritis. Clinically, patients present with typical symptoms such as cough, dyspnea and chest discomfort. However, the radiologic manifestations in patients are highly prominent, including pulmonary nodules, pleural thickening, ground-glass opacities and interstitial lung disease. A characteristic lesion of IgG4-RD, the bronchovascular bundle, is also identified radiologically through a CT scan².

The pathological differences in the lung and other affected organs contrast strikingly. Lungs tend to exhibit obliterative arteritis, which is rarely evident in other organs (as well as the more common obliterative phlebitis). Further, lungs are more likely to have neutrophilic infiltrate, due to their interface with the external environment.

Pancreas

The pancreas displays the typical histopathological features of IgG4-RD in patients with *type 1* autoimmune pancreatitis (AIP). This form of AIP is more prevalent worldwide and is associated with IgG4, unlike type 2 AIP, which is unrelated to IgG4 and rather features neutrophilic infiltration into the epithelium of the pancreatic duct.

Interestingly, the pancreas was one of the first organs to be associated with elevated serum IgG4 levels. However, evaluation of additional criteria including pancreatic histology, imaging and glucocorticoid responsiveness is necessary to differentiate autoimmune pancreatitis from pancreatic cancer. This is vital, as many patients have undergone unnecessary surgical procedures due to presumed pancreatic cancer. Indeed, there are several reported cases of pancreatic cancer in patients with AIP.

Other organs

The systemic nature of IgG4-RD adds much diversity to its clinical presentations; we are now seeing involvement of organs previously thought to be out of the IgG4-RD spectrum. For example, although brain involvement is rare, IgG4-RD is the most common causes of hypertrophic pachymeningitis. Similarly, skin manifestations such as erythematous papules and hyperpigmented lesions have been reported in the head and neck areas.

These features display the complexities and multi-faceted nature of IgG4-RD and suggest a complicated disease pathogenesis consisting of multiple pathways.

Histopathology of IgG4-Related disease

The systemic nature of IgG4-RD has prevented the recognition of an underlying condition for centuries and its mimicry of conditions, ranging from inflammatory disorders to malignancies,

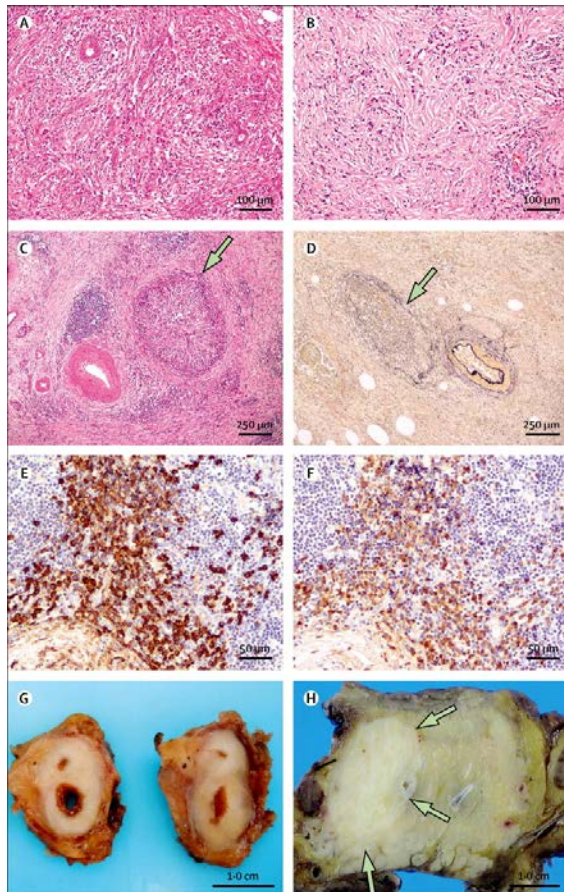


Figure 2. Pathological feature of IgG4-RD.
*Picture Credit: Kamisawa et. al*²

has further helped in its disguise. However, the identification of common histopathological and immunohistochemical features linked disorders once thought to be single-organ conditions. Today, an accurate, and definitive diagnosis can be achieved through a histopathological interpretation. This contrasts most autoimmune conditions that are diagnosed through a serological evaluation, such as rheumatoid arthritis. A tissue biopsy is the gold standard for a diagnosis as it reveals the three central features of IgG4-RD²:

Storiform Fibrosis

All IgG4-RD patients exhibit some extent of storiform fibrosis (figure 2B). Patient tissues display an abnormal pattern of radially arranged collagen fibers, which is thought to be driven by macrophages and activated fibroblasts. This form of fibrosis is characteristic to IgG4-RD and is rarely seen in other inflammatory diseases. Despite its characteristic appearance, it typically appears in patches and thus can often be overlooked.

Obliterative Vascular Disease

Obliterative vascular disease is the partial or complete occlusion of blood vessels by inflammatory infiltrate comprised of lymphocytes and plasma cells. Veins are predominantly affected in IgG4-RD, with patients exhibiting extensive obliterative phlebitis (figure 2C). These veins commonly form an inflammatory nodule next to a patent artery, which is easily identified from a tissue biopsy (figure 2H). Although rare in most organs, arteries can also be affected, and is seen most commonly in the lungs. Both obliterative phlebitis and obliterative arteritis are unique to IgG4-RD, and thus carry immense diagnostic value.

Lymphoplasmacytic Infiltrate

IgG4-RD can be distinguished by the abnormally high IgG4-positive plasma cells in patient tissues. Although IgG4 concentrations in patient sera are not always elevated, they are notably high across patient lesions. This hallmark alone is not sufficient to determine a definitive diagnosis, as IgG4-positive cells are present in inflammatory and neoplastic disorders such as granulomatosis with polyangiitis, rheumatoid arthritis, etc. Thus, additional criteria need to be assessed in order to confirm disease diagnosis, such as the ratio of IgG4:IgG+ plasma cells (Figure 2F).. Also seen elevated in lesions are plasma cells expressing other immunoglobulins and subtypes, albeit in concentrations much lower than IgG4-bearing plasma cells.

Pathophysiology

Role of Immunoglobulin G4

Despite the elevated tissue, and occasionally serum, concentrations of IgG4 it is unlikely that this immunoglobulin is the driver of pathogenesis. Unlike the other subsets of IgG, IgG4 is unable to fix complement as it undergoes a process called Fab arm exchange. Here, the heavy chains dissociate into two hemi-molecules, which then associate with other hemi-molecules. Thus, IgG4 antibodies are functionally monovalent and unable to bind to activating Fc γ R. Rather, they are thought to be anti-inflammatory as they bind well to inhibitory Fc γ R. Although this has been the conventional thinking, recent literature illustrates the pathogenicity of serum IgG4 (from patients with AIP) on mice pancreas and salivary glands.

Current hypothesis

The immune response seen in IgG4-RD is complex, with inflammatory lesions infiltrated with high levels of characteristic lymphocytes (IgG4+ plasmablasts, CD4+ Cytotoxic T-cells) as well as innate immune cells (macrophages, eosinophils, fibroblasts, and myofibroblasts). There are two processes that are thought to result in the clinical manifestations of IgG4-RD.

The first implicates the role of T helper cells in disease pathogenesis, which seems plausible due to their high concentration and localization to patient lesions. These T helper cells are polarized in response to an unknown trigger such as commensal microbes, environmental allergens, infectious pathogens or even tissue damage (whether the Th1 or Th2 pathway is activated remains disputed). Once polarized, these T helper cells produce a robust inflammatory cytokine

milieu that includes IFN- γ , IL-4, IL-5 and IL-13. Further, T-follicular helper cells produce IL-4 and IL-10 to drive preferential class switching of autoreactive B cells to IgG4 and IgE, and thus induce the differentiation and expansion of IgG4+ plasma cells³.

The cytokine milieu further shapes IgG4-RD into a *fibro*-inflammatory disorder by generating profibrotic macrophages, while Interleukins 5 and 13 and TGF- β recruit eosinophils and activate fibroblasts. Both the macrophages and fibroblasts then create the dense storiform pattern of fibrosis that is characteristic of IgG4-RD³.

The second underlying process is thought to be a feedback negative regulatory process involving autoreactive plasmablasts that present self-antigen to autoreactive T cells. This leads to a cycle of T-B cell collaboration and increasing numbers of high-affinity autoreactive B cell clones, finally resulting in the fibroinflammatory immune response seen in IgG4-RD patients².

Pathogenesis

Although IgG4-RD is a relatively new entity, recent research has elucidated key players in its pathogenesis. Recent publications from the Pillai Lab have identified two characteristic cell types that were largely increased in active IgG4-RD patients.

Expanded CD4+CTLs cells have a restricted TCR β -repertoire

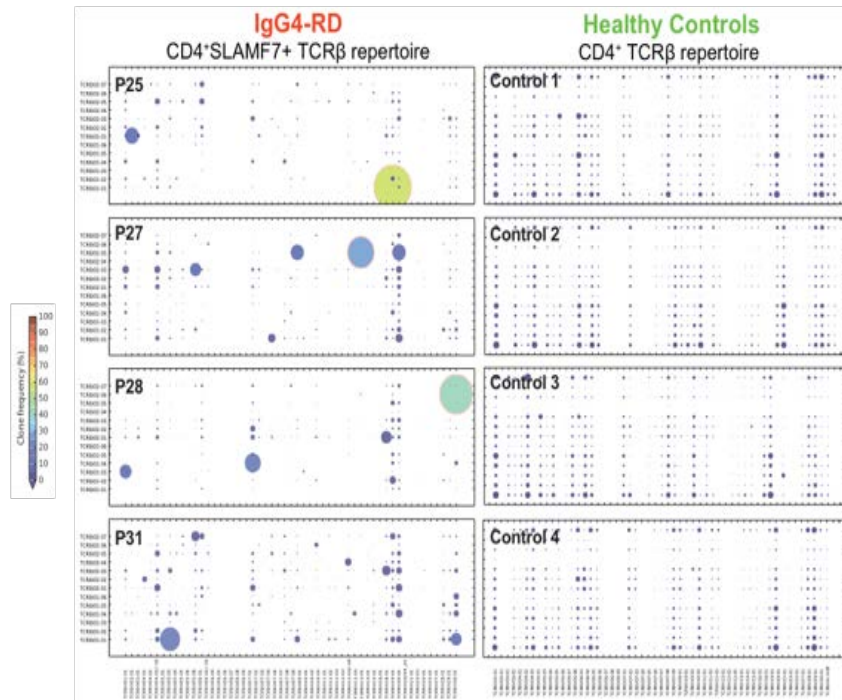


Figure 3. TCR β repertoire of circulating expanded T effector memory cell subset in 4 IgG4-RD patients. *Figure from Matoo et. al¹*

The first, CD4+ cytotoxic T lymphocytes, were clonally expanded and infiltrated affected tissue sites. The clonality of these expanded cells was identified by next generation sequencing of the T cell receptor β chain, revealing a predominance of SLAMF7, granzymeA, IL-1 β and TGF- β expressing CD4+ CTL's. ¹

The second lymphocyte population of CD19+ CD27+ CD20- CD38^{hi} cells is specifically interesting, as they are a hallmark of active IgG4-RD. These clonally expanded plasmablasts undergo enhanced somatic hypermutation, express high levels of MHC II molecules and are IgG4+. Interestingly, Mattoo et. al⁴ further illustrated that these plasmablasts secrete self-reactive IgG4 autoantibodies.

Patients treated with B cell depletion therapy using Rituximab, an anti-CD20 monoclonal antibody, showed a striking clinical improvement. Most importantly, patients exhibited a clear correlation between clinical improvement and decreased CD4+ CTL's (and of course B-cell depletion)⁵. This correlation, as well as the high MHC II expression on these self-reactive plasmablasts, alludes to their role as antigen presenting cells to CD4+ CTL's. Whether they present antigen to trigger initial disease or in the later stages to simply reactivate CD4+ CTL's remains unknown. However, the key question that will help elucidate diseases pathogenesis is:

What autoantigens are these self-reactive plasmablasts presenting?

I sought to approach this challenge from multiple directions. First, I would identify the primary autoantigen using an elegant technique known as PLATO. In addition, I would also study autoantigens that had previously been identified in autoimmune pancreatitis (AIP)—now considered a manifestation of IgG4-RD. These autoantigens had not been previously looked at in the context of IgG4-RD, thus studying them in a heterogeneous IgG4-RD cohort would potentially provide great insight.

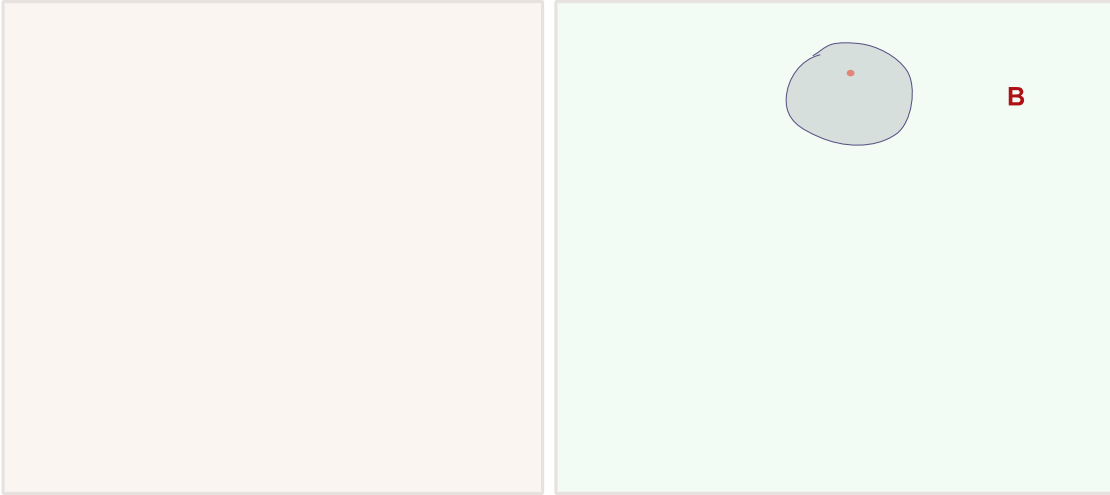


Figure 4. Schematic diagram summarizing pathogenesis in IgG4-RD. *Picture Credit:*
Shiv Pillai

Chapter Two

Data: Short Introduction

Studies over the past decade have provided great insight into the immune dysregulation in IgG4-RD, particularly the identification two characteristic lymphocytes that are clonally expanded in active patients: CD19⁺ CD27⁺ CD20⁻ CD38^{hi} plasmablasts¹ and CD4⁺ cytotoxic T lymphocytes⁴. We believe these plasmablasts are presenting antigens to CD4⁺ CTL's, identifying the specific autoantigens is key in elucidating disease pathogenesis and hopefully, the etiology of IgG4-RD.

Experiment Outline

Here, we used an elegant technique, PLATO, to identify immune targets of serum antibodies from patients with active IgG4-RD. We sought to validate these PLATO interactions after determining the top target autoantigens, and cloned plasmids with genes encoding the top twenty proteins. These plasmids were transfected into suspended 293F cells to produce large-scale recombinant proteins. After two days, cells were lysed and the protein of interest was purified using an affinity column. Lastly, these recombinant autoantigens were used to test specificity of serum autoantibodies using an indirect ELISA assay.

Materials and Methods

Parallel analysis of translated Open Reading Frames (PLATO)

First published in 2013, PLATO is an elegant *in vitro* technique designed by the Stephen Elledge to discover protein-protein interactions in an unbiased manner⁶. First, PLATO transcribes and translates a library of open reading frames (ORFs) *in vitro*. Then using Ribosomal Display, ORF-encoded proteins and mRNA are then linked into a complex— which remains intact due a mutated stop codon— and exposed to surface-immobilized ‘bait’ molecules, such as antibodies. Finally, bound mRNA is eluted, reverse transcribed and sequenced for protein identification. This experimental protocol can identify novel proteins for various uses, such as drug target determination, protein-protein interaction and also for identifying autoantibody antigen. PLATO has several advantages over other screens, namely library size and cost. Being an *in vitro* technique further provides advantages such as the ability to synthesize proteins toxic to cells, but can also serve as a limitation, as *in vitro* proteins lack the natural conformation and post-translational modifications seen *in vivo* proteins.

PLATO overview:

The Elledge Lab at Harvard Medical School performed PLATO and provided us with the top protein ‘hits’ shared in Figure 5. However, a review of the experimental design from protocol is shared below⁶:

1- Preparation of the Ribosomal Display ORFeome library

PLATO requires a library of ORF's as input, thus we utilized the human ORFeome collection, which was constructed via PCR cloning from cDNA libraries into the Gateway entry vector system. Importantly, native stop codons were mutated into coding sequences to allow addition of the C terminal fusion tags to expressed polypeptides (Zhu et. al 2013). To avoid representational bias such as lower recombination and amplification efficiencies in longer transcripts, the ORFeome entry vector library was split into 18 subpools and subcloned into the pRD-DEST expression vector. Expression plasmids were then PCR-amplified, resulting in DNA templates ready for *in vitro* transcription.

2- 'Panning': Library enrichment on surface-immobilized bait

Ribosomal display (RD) was then employed to link the ORF-derived mRNA molecules to their encoding proteins, and the complex of mRNA-protein-ribosomes was incubated with serum autoantibodies to undergo 'panning'. These antibodies were immobilized as they were covalently bound to magnetic protein A/G beads.

3- Conversion of recovered mRNA into 3' tag DNA libraries for deep sequencing or qPCR analysis.

The enriched mRNA library is then eluted, fragmented and reverse transcribed. The 3' end of the resulting cDNA is then polyadenylated prior to deep DNA sequencing and analysis. We further subjected the enriched mRNA library to ORF-specific qPCR to validate PLATO interactions.

Gateway Cloning

We then sought to synthesize the top twenty proteins identified by PLATO and test them against patient serum. In order to do so, we purchased Gateway vectors expressing genes that encoded these proteins. We then cloned our genes of interest into the destination vector pHWF using Invitrogen™ Gateway™ recombination cloning.

Subjects

Sera from 100 IgG4-RD patients were studied and compared to 40 healthy donors. In addition, a disease control cohort consisting of 6 Systemic lupus erythematosus (SLE), 9 Anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) and 15 Rheumatoid Arthritis (RA) patients was added to evaluate disease specificity. We used a heterogeneous cohort of IgG4-RD patients who expressed a range of clinical manifestations. Results were later analyzed based on patient labs and organ manifestations.

Cell Culture and Transfection

We initially cultured and transfected Human Embryonic Kidney (HEK) 293T cells to express our proteins of interest. However, we later optimized our protocol to incorporate large-scale protein production and used suspended 293 Freestyle cells (ThermoFisher catalog no. R79007). These cells were cultured at a concentration of 0.5×10^6 cells/ml in 250ml of Freestyle medium (ThermoFisher catalog no. 12338018) and placed in an orbital shaker incubator at 37 °C, 120 rpm, and 5% CO₂ for 24 hours. Cells were then co-transfected with protein encoding plasmids

and PEI and placed back in the orbital shaker incubator for an additional 48 hours. Finally, the transfected cells were centrifuged for 5 minutes at 3,000x, and the resulting pellets were stored at -80°C (if not immediately lysed).

Cell lysis and protein purification

The pellets obtained from centrifugation were then lysed using with our lysis buffer (100 mM potassium acetate, 50 mM Tris pH 7.5, 1% NP-40, protease inhibitors). We further ensured complete cell lysis by sonicating cells for 5 cycles (15 seconds on and off, at 10 Hz amplitude). Cell lysates were then spun at 3,000x g at 4°C for 25 minutes to remove cell debris; the supernatant was stored for purification.

Proteins were then purified using affinity chromatography, as our desired proteins were all FLAG tagged. Supernatant was incubated overnight at 4°C with anti-FLAG affinity beads (Sigma Aldrich cat. no. A2220), and then run through a column. Proteins were then eluted via competitive binding, by running 5 column volumes of 100 µg/ml of FLAG peptide (Sigma Aldrich cat. no. F3290)

All samples were run on a 10% SDS-PAGE gel followed by an anti-FLAG western blot to confirm we isolated our protein of interest.

ELISA (Enzyme Linked Immunosorbent Assay)

Nunc-Immuno™ MicroWell™ 96 well solid plates (Sigma Aldrich cat. no. M9410) were coated with 100µl of 5µg/ml antigen and incubated overnight at 4°C. Wells were then washed 4 times with phosphate buffered saline containing 0.05% Tween-20 (PBS-T) and then incubated overnight at 4°C with 300µl of 5% bovine serum albumin in PBS-Tween. After four washes of PBS-T, patient serum (1:100) was added to wells and left to incubate at room temperature for 2 hours. Wells were washed with PBS-T four times and then incubated with secondary antibody (anti-IgG conjugated with horseradish peroxidase enzyme (Sigma Aldrich cat. no. A8792) for one hour at room temperature. After four more washes of PBS-T, 100µl of 0.4mg/ml OPD substrate (Sigma Aldrich cat. no. P9187) was added to wells and left to incubate at room temperature for 15 minutes. The reaction was then stopped using 100µl of 3M sulfuric acid solution and absorbance was determined at 492nm. All wells were normalized to blank wells (containing only PBS), and absorbance due to antigen was calculated by subtracting absorbance of control wells (with patient sera but no antigen) from antigen-coated wells. All samples were performed in triplicates.

Statistical Analysis

The Fischers exact test was used to evaluate differences between antibody responses in IgG4-RD patients compared to those in the control group (healthy donors), and also to analyze correlations between specific autoantigens and organ involvement. Statistical difference was defined as $p < 0.05$.

RESULTS

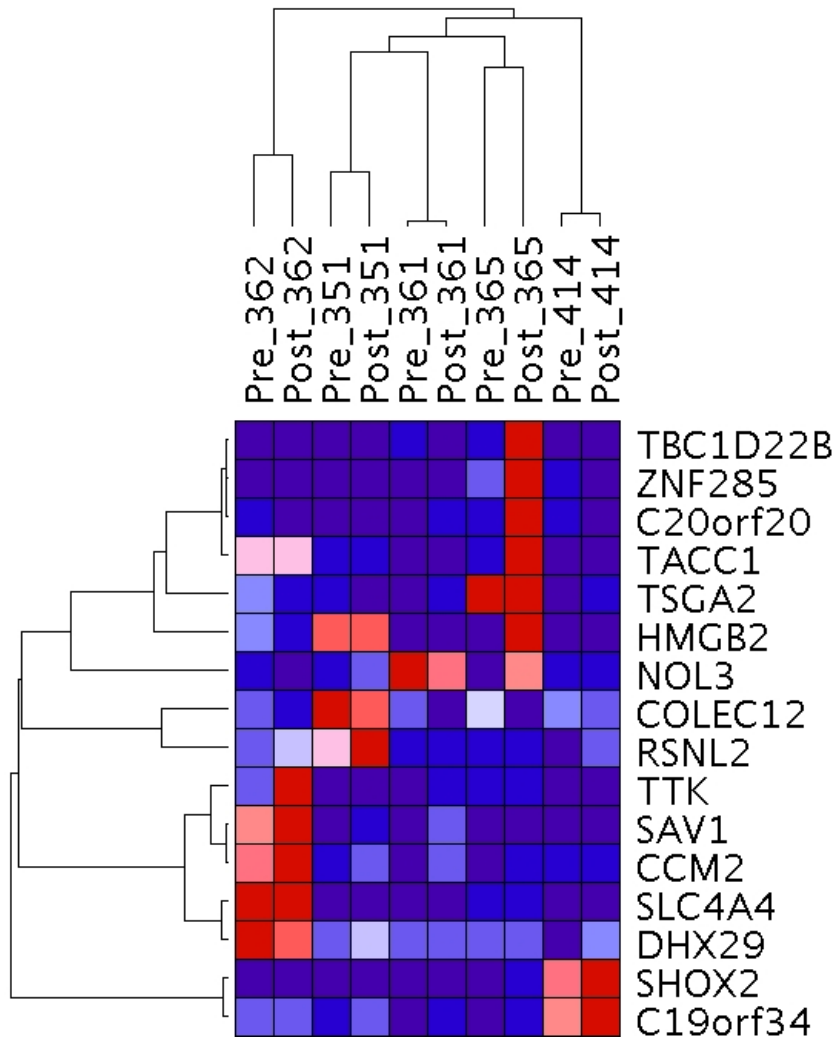


Figure 5. Heat map illustrating PLATO results. Red indicates hits with >3.5 fold enrichment compared to healthy controls, intense red indicates a higher number of hits (Range- 3.5x -19x).

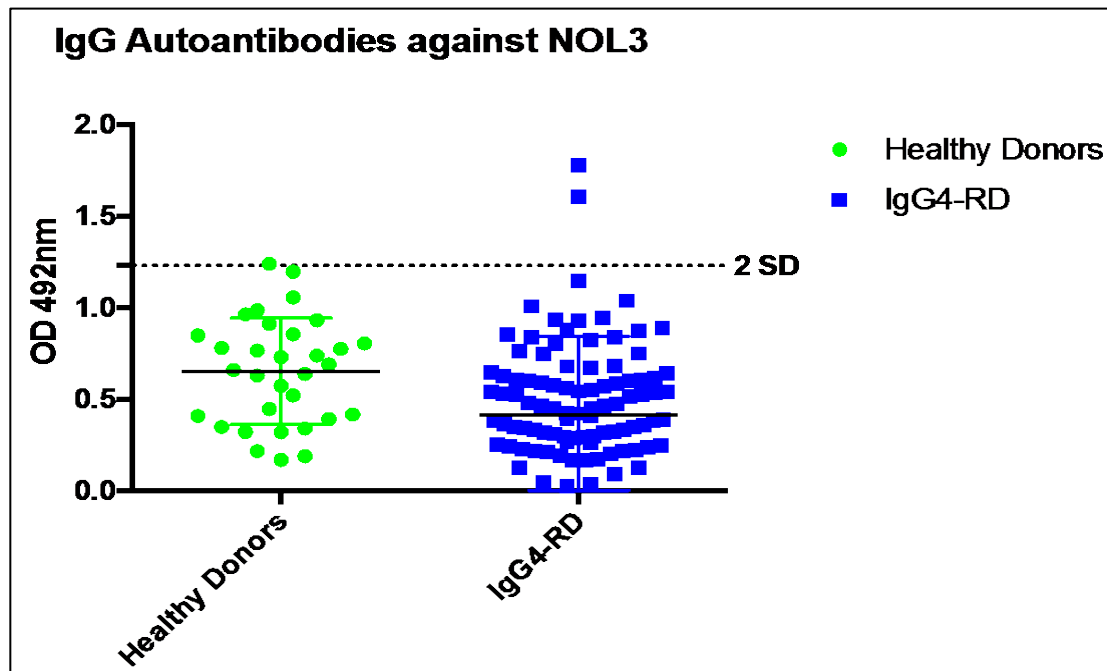


Figure 6. Antibody specificity against NOL3. Setting a stringent cutoff at 2 SD above the mean of healthy donors results in 2 % of IgG4-RD patients as positive. Interestingly, both positive patients exhibited midline soft tissue fibrotic lesions (i.e. 10% of this subgroup is specific to NOL3).

Autoantigens identified in autoimmune diseases other than IgG4-RD

1. Lactoferrin

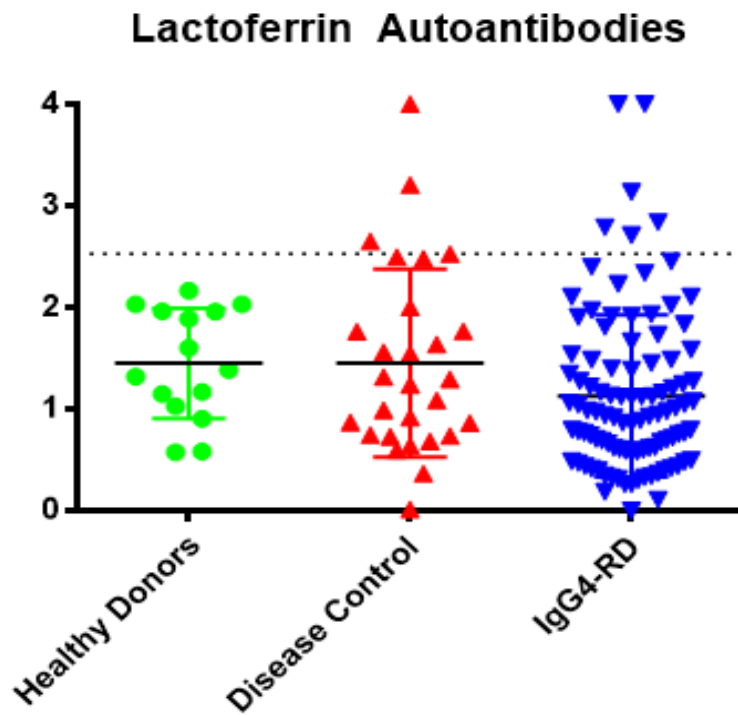


Figure 7. Absorbance of anti-lactoferrin IgG autoantibodies. A stringent cutoff at 2 SD above the mean of healthy donors was set, noting 6% of IgG4-RD patients as positive.

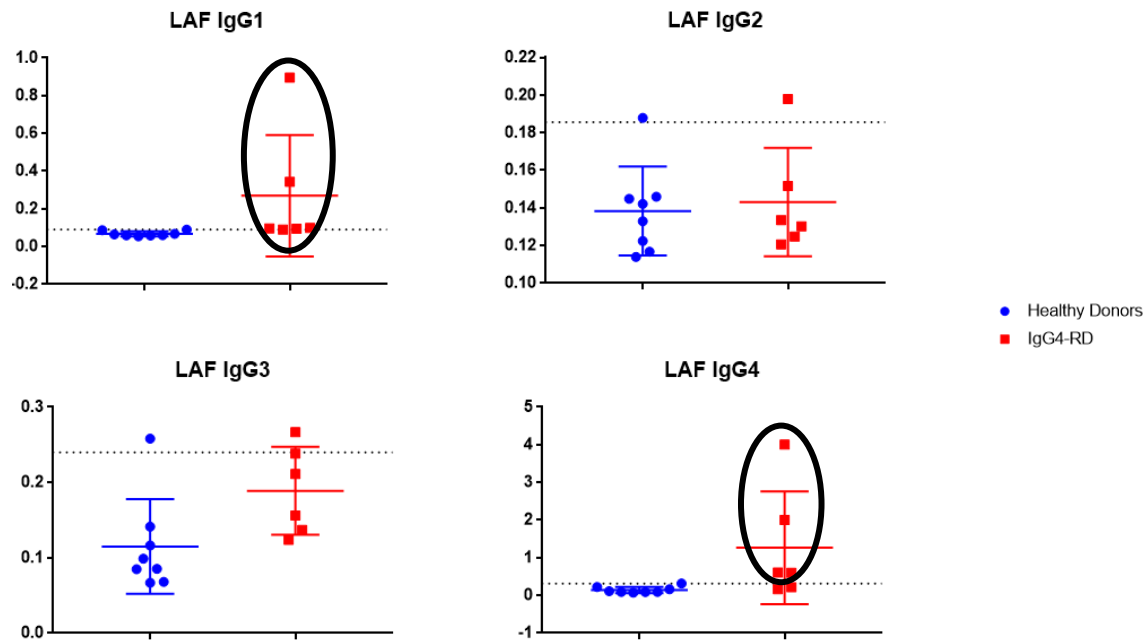


Figure 8. IgG subclass specificity to Lactoferrin. Patients positive in Fig. 7 were tested against each IgG subclass, showing the presence of anti-lactoferrin IgG1 and IgG4 antibodies.

2. Carbonic Anhydrase 1

Autoantibodies against Carbonic Anhydrase 1

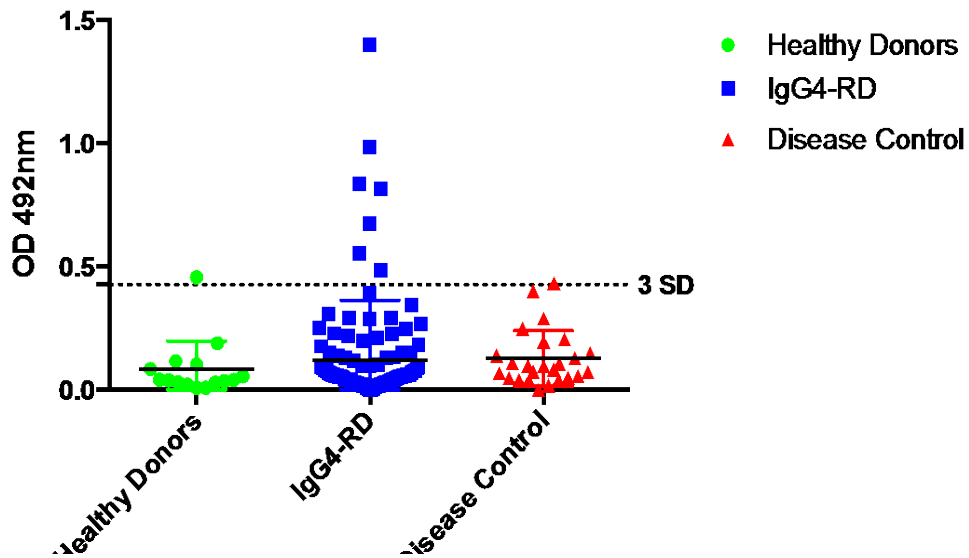


Figure 9. Absorbance of anti-CA1 IgG autoantibodies. A stringent cutoff at 3 SD above the mean of healthy donors was set, noting 7% of IgG4-RD patients as positive.

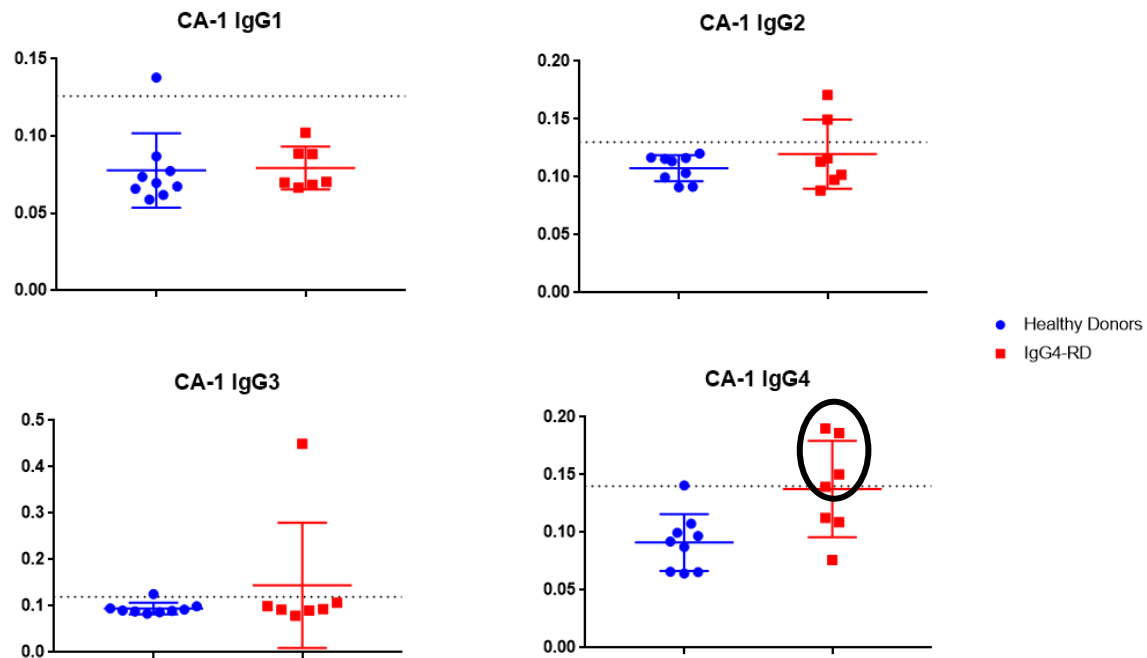


Figure 10. IgG subclass specificity to Carbonic Anhydrase 1. Patients positive in Fig. 9 were tested against each IgG subclass, showing the presence of anti-CA1 IgG2 and IgG4 antibodies.

3. Carbonic Anhydrase 2

Autoantibodies against Carbonic Anhydrase 2

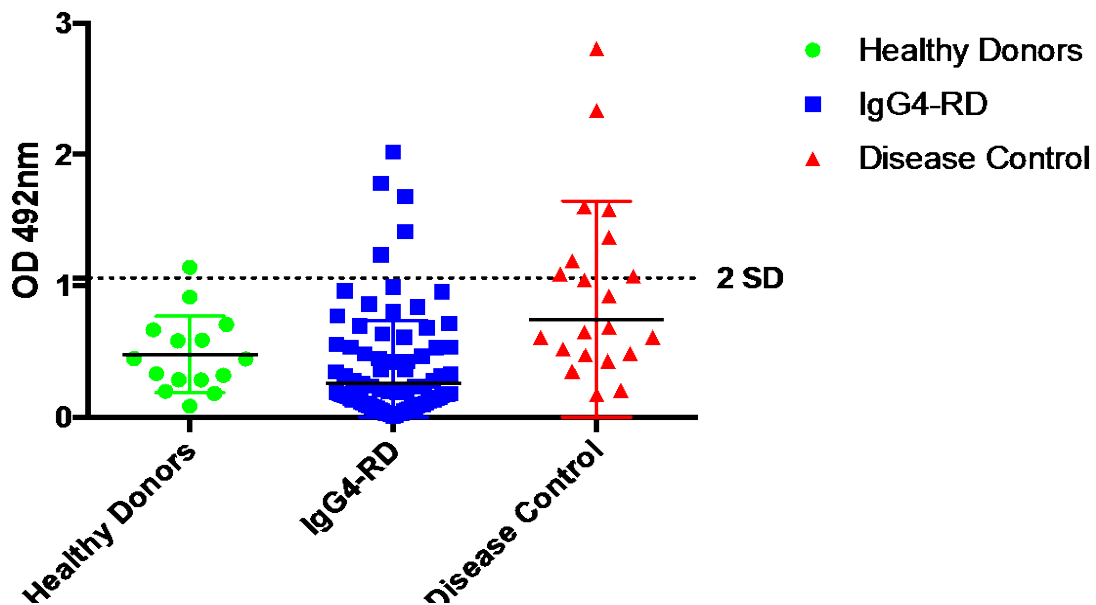


Figure 11. Absorbance of anti-CA2 IgG autoantibodies. A stringent cutoff at 2 SD above the mean of healthy donors was set, noting 5% of IgG4-RD patients as positive.

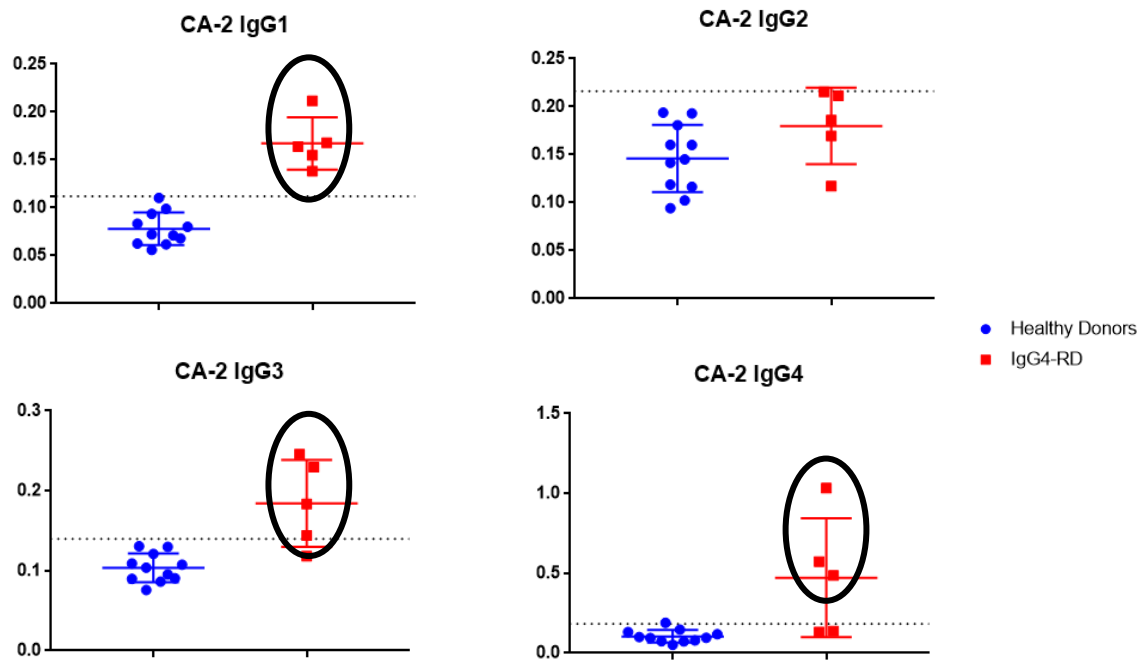


Figure 12. IgG subclass specificity to Carbonic Anhydrase 2. Patients positive in Fig. 11 were tested against each IgG subclass, showing the presence of anti-CA2 IgG1, IgG3 and IgG4 antibodies.

4. Carbonic Anhydrase 4

Autoantibodies against Carbonic Anhydrase 4

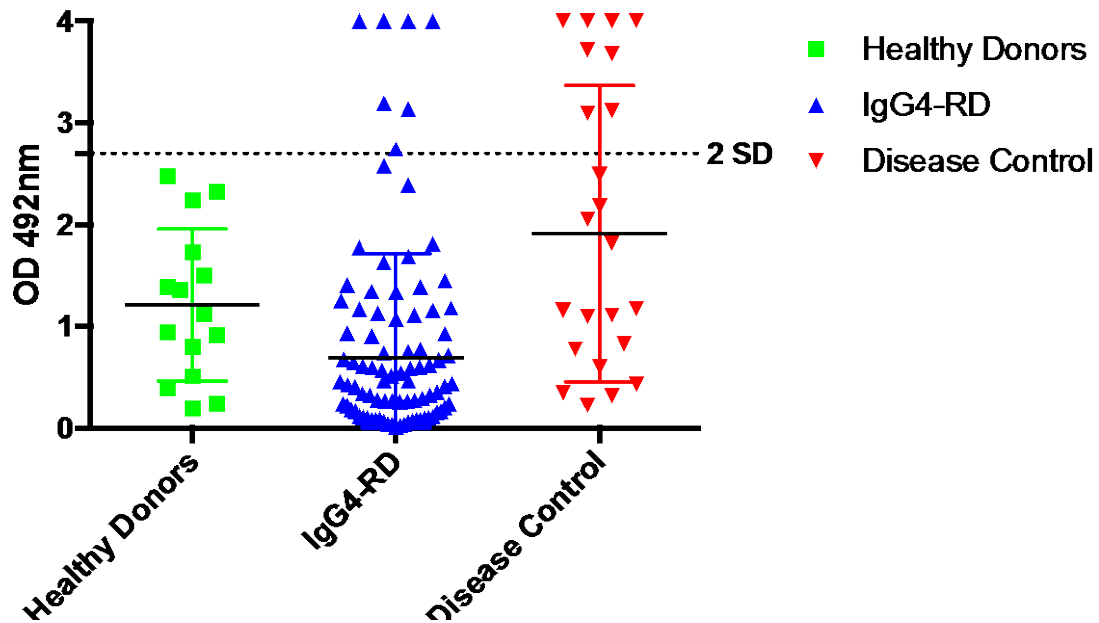


Figure 13. Absorbance of anti-CA4 IgG autoantibodies. A stringent cutoff at 2 SD above the mean of healthy donors was set, noting 7% of IgG4-RD patients as positive.

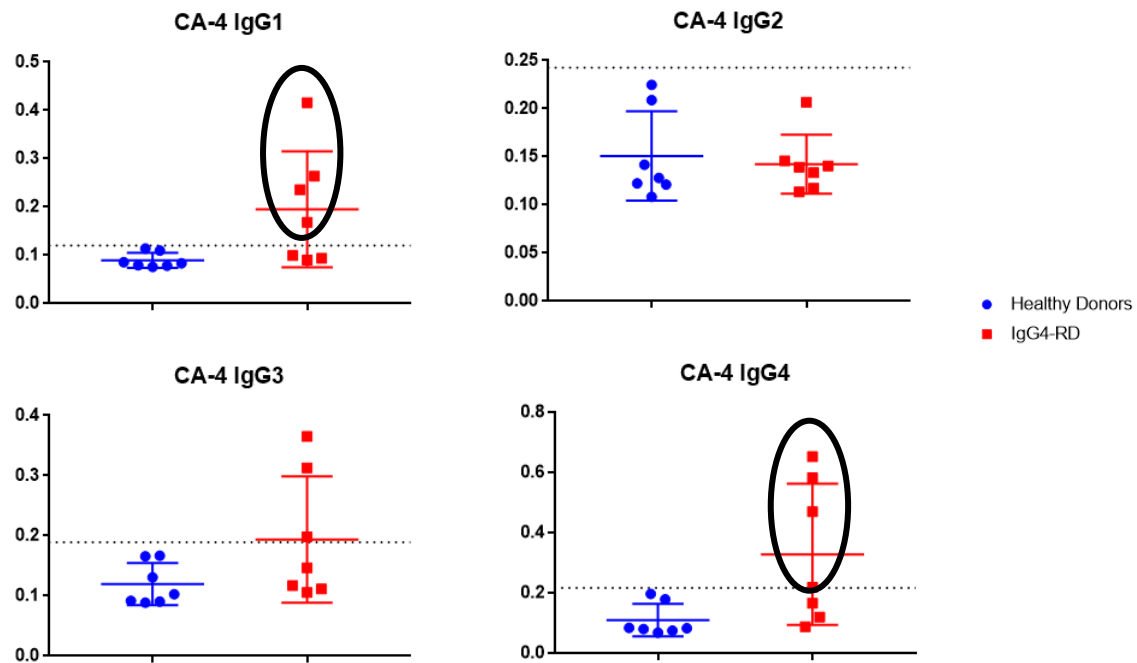


Figure 14. IgG subclass specificity to Carbonic Anhydrase 4. Patients positive in Fig. 13 were tested against each IgG subclass, showing the presence of anti-CA4 IgG1 and IgG4 antibodies.

Pancreatic Autoantigens

5. Proteinase Serine 2 (PRSS2)

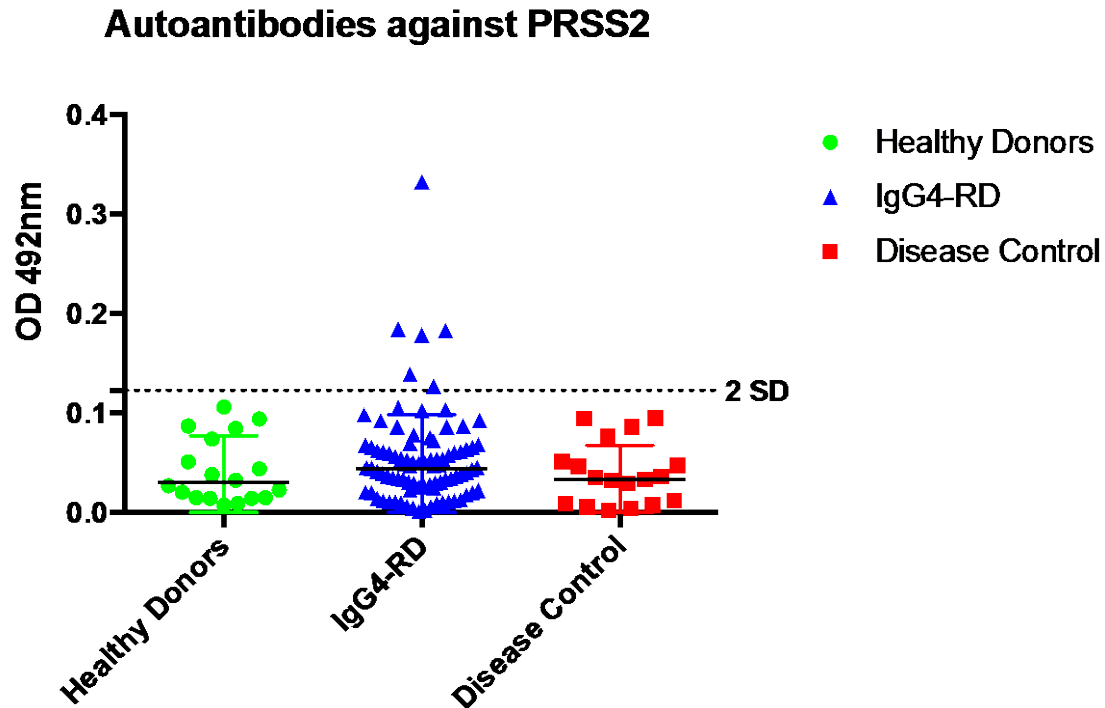


Figure 15. Absorbance of anti-PRSS2 IgG autoantibodies. A stringent cutoff at 2 SD above the mean of healthy donors was set, noting 6% of IgG4-RD patients as positive. Notably, no disease controls showed anti-PRSS2 IgG thus PRSS2 seems to be disease specific.

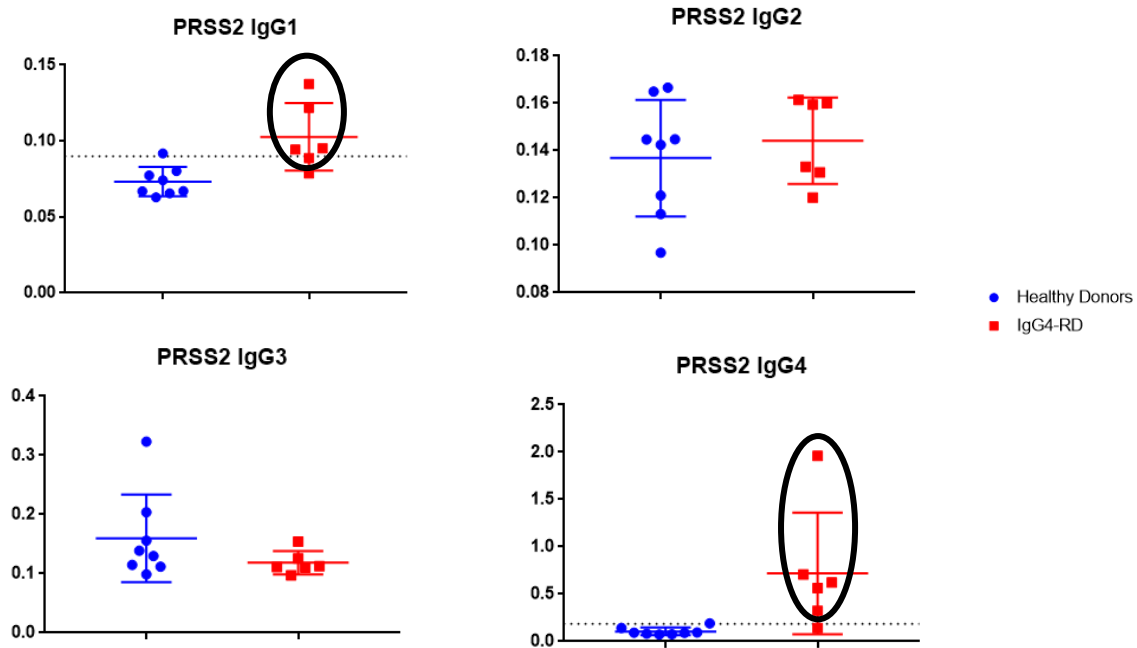


Figure 16. IgG subclass specificity to PRSS2. Patients positive in Fig. 15 were tested against each IgG subclass, illustrating the presence of autoreactive IgG1 and IgG4 antibodies.

6. Pancreatic secretory trypsin inhibitor (SPINK-1)

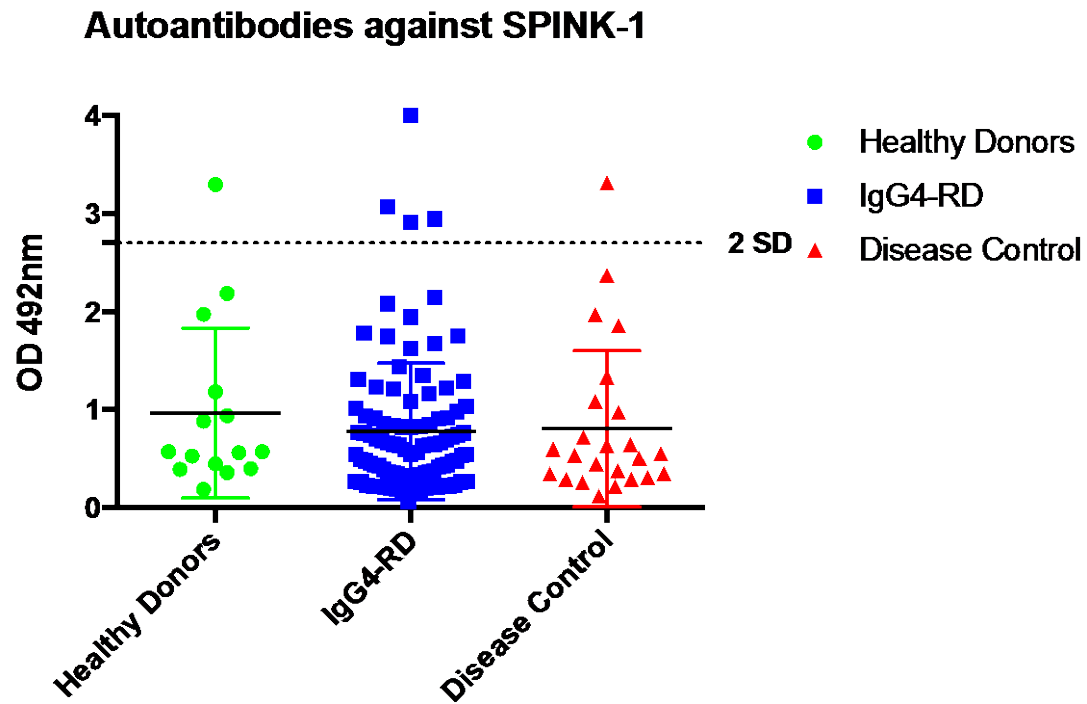


Figure 17. Absorbance of anti-SPINK-1 IgG autoantibodies. A stringent cutoff at 2 SD above the mean of healthy donors was set, noting 4% of IgG4-RD patients as positive.

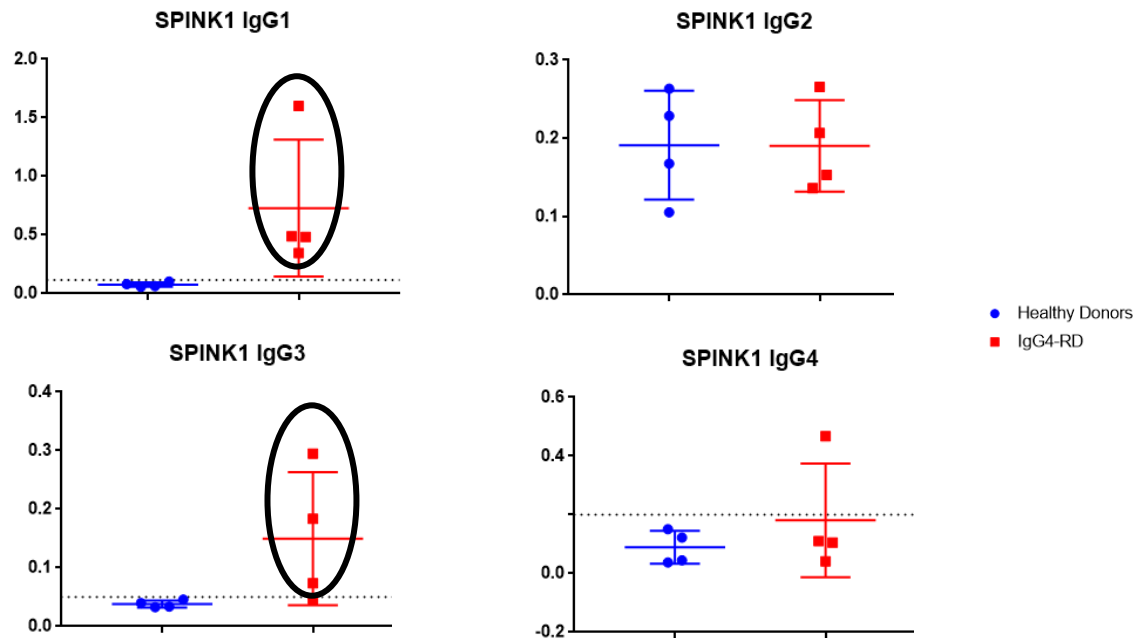


Figure 18. IgG subclass specificity to SPINK-1. Patients positive in Fig. 17 were tested against each IgG subclass, showing the presence of autoreactive IgG1 and IgG2 antibodies.

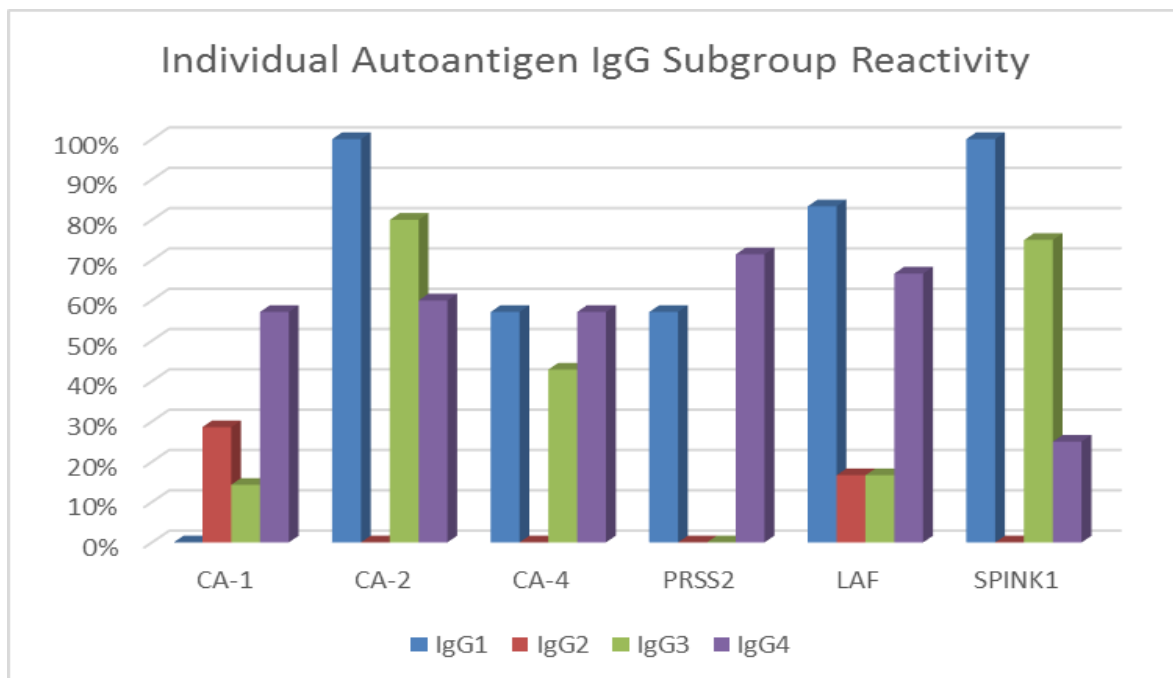


Figure 19. Chart summarizing IgG subclass specificity to autoantigens.

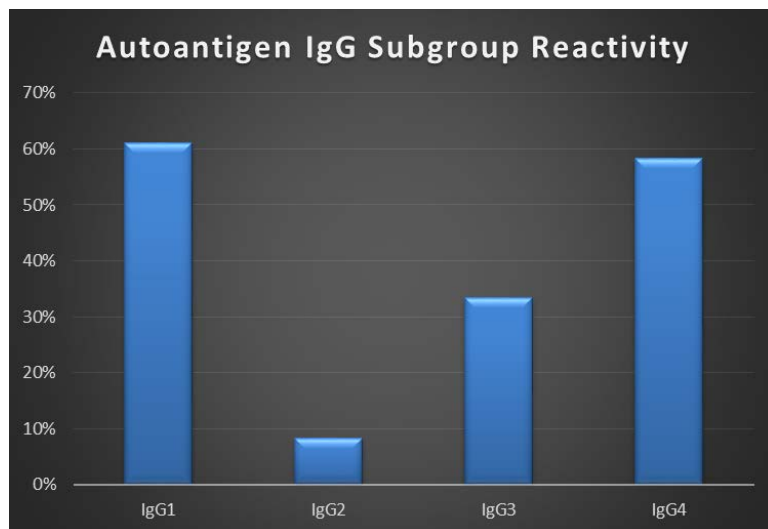


Figure 20. Predominance of autoantigen specific IgG1 and IgG4 subclasses.

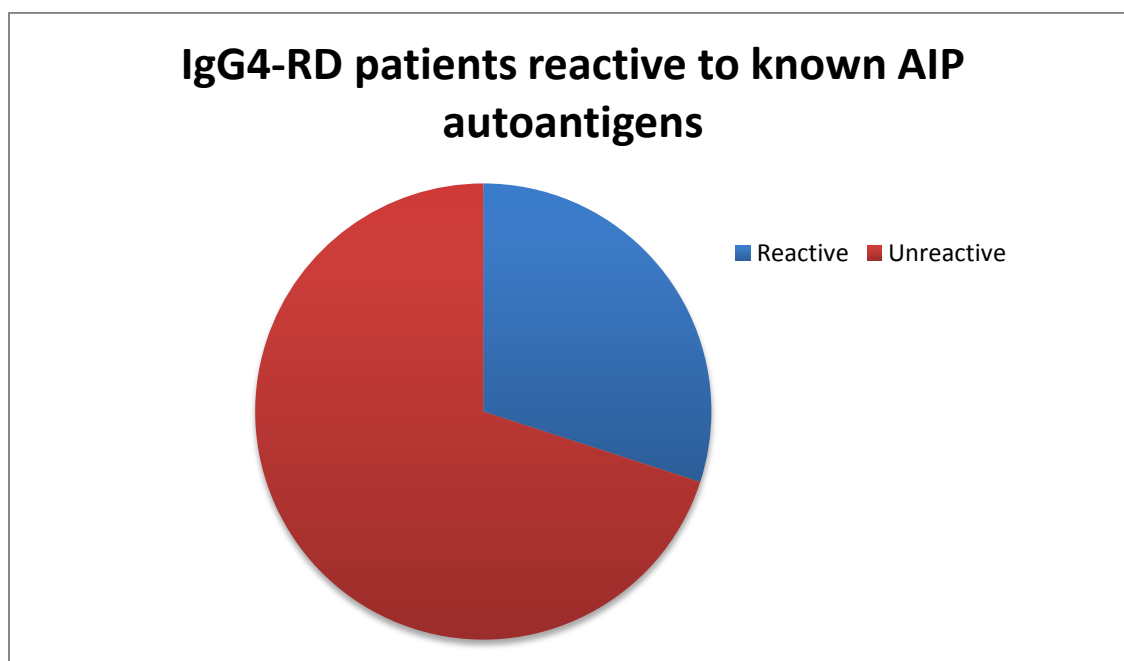


Figure 21. 33% of patients were reactive to at least one of the known autoantigens in AIP. Only 7 of 33 are AIP patients, thus these autoantigens are not limited to AIP but also present in other organ manifestations in IgG4-RD.

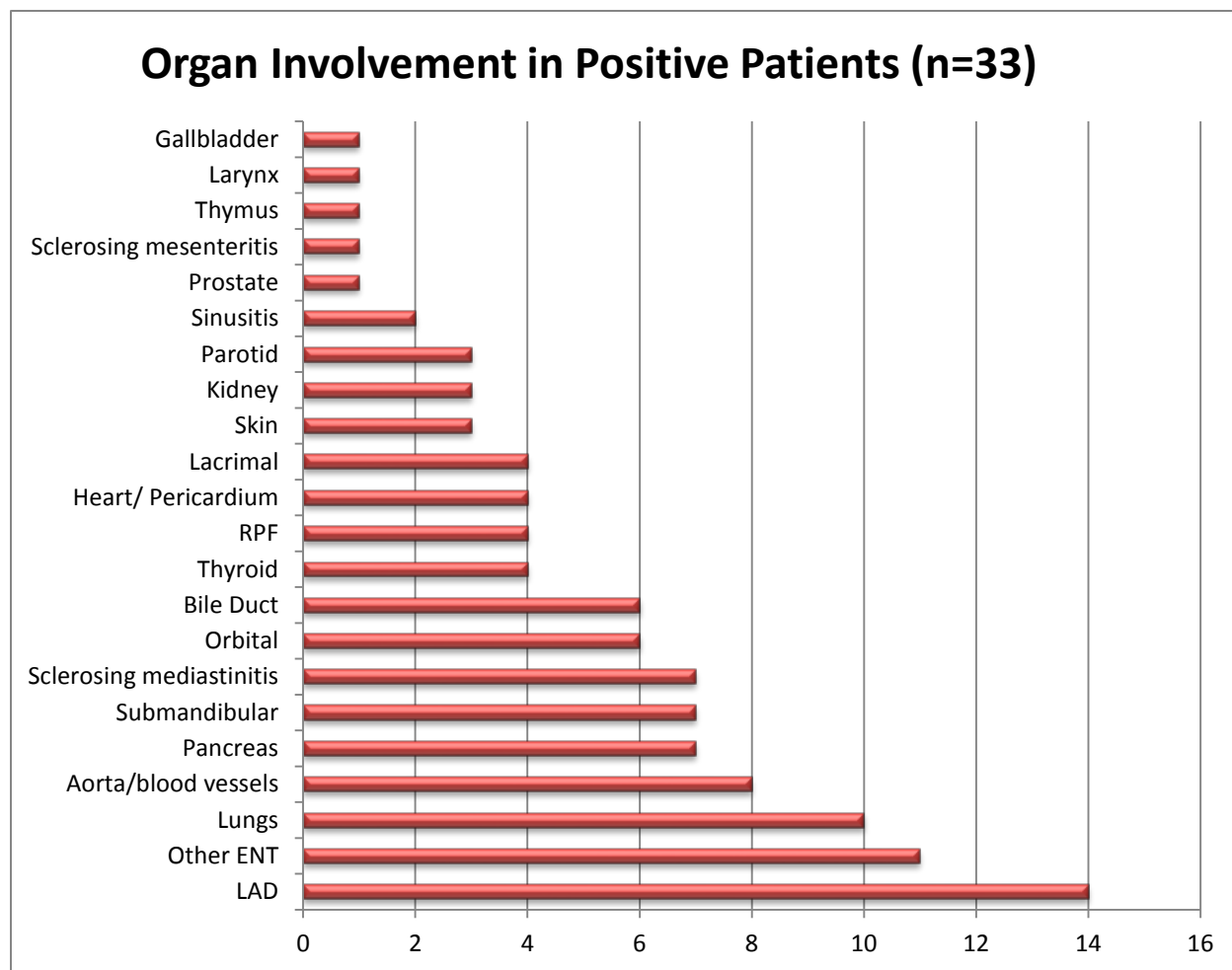


Figure 22. Autoantigen reactivity is not limited to AIP patients. Varying organ manifestations in positive patients. Particularly interesting was lung involvement; no previous studies shown a correlation between lung involvement and increased autoantigen specificity.

Organ Involvement in Positive Patients (n=33)	
Other ENT	p<0.01
Lungs	p<0.01
Sclerosing mediastinitis	p<0.008
Submandibular	p<0.25
Parotid	p<0.77
Pancreas	p=1
Orbital	p=1

Figure 23. Fischer exact test results depicting correlation of autoantigens and organ manifestations in positive patients. Autoantigen reactivity correlates with manifestations of Lungs, Sclerosing Mediastinitis and Other ENT involvement.

Variable (mean)	Auto-Ab Positive (n=26)	Auto-Ab Negative (n=74)	p-value
Total IgG	2,035	1,823	0.56
IgG1	1,045	917	0.36
IgG2	665	695	0.89
IgG3	83	162.8	0.14
IgG4	548	534	0.89
IgE	342	505	0.41

Figure 24. No lab correlations with positive patients.

Brief Discussion

Interestingly, our ELISA data revealed the presence of multiple autoantigen specific IgG subclasses in positive patients, as well as the predominance of IgG1 and IgG4. The elevated subclasses seen in positive patients correlated with serum subclass elevations evident in lab reports (data not shown). These findings may allude to a possibility of interplay between the IgG subclasses, i.e. IgG1 may be driving disease pathogenesis while the non-inflammatory IgG4 molecule may be clonally expanded to suppress the inflammatory response.

Previous literature on autoantigens in diseases such as SLE and RA indicates the loss of tolerance, with many autoimmune conditions sharing the same autoantigens⁷. This can be seen in our ELISA results, where disease controls also exhibit autoreactive IgG to antigens such as Carbonic Anhydrases 2 and 4. However, other autoantigens tested seemed to be more specific to IgG4-RD, such as Carbonic Anhydrase 1, PRSS2 and SPINK-1.

Limitations

Although we successfully identified patient specificity to the above autoantigens, our experiment had some limitations. We synthesized our recombinant PLATO proteins and verified their presence via western blot but were unable to detect them on a Coomassie stain. This meant we had not synthesized a sufficient yield, which may have been due to an unsuitable plasmid or early degradation of our desired protein. Although this hindered our ability to test all PLATO

proteins, we still analyzed specificity of one PLATO protein, NOL3, after purchasing it commercially.

Furthermore, we are currently utilizing an alternative, highly sensitive antibody-profiling assay – Luciferase Immunoprecipitation System (LIPS) to detect specificity to PLATO proteins⁸. We are also cloning our genes of interest into a new plasmid encoding a secretory signal, to optimize protein production and increase protein yield.

Discussion and Perspectives

Antigen presentation plays a central role in most, if not all, autoimmune diseases. It is no coincidence that the major histocompatibility complex gene is a major disease susceptibility locus in numerous autoimmune conditions such as Multiple sclerosis, Rheumatoid Arthritis, and Type-1 diabetes⁷. A growing number of studies on the peptide-MHC-TCR interaction in these diseases have identified a wide range of autoantigens, some characteristic of a particular disease, while others are shared across many diseases⁹.

Further, it is also becoming increasingly evident that antibodies play a vital role in the pathogenesis of such diseases, from tissue injury to exacerbating systemic inflammation⁷. The role of B cells in autoimmunity has been studied in many diseases such as MS and now in IgG4-RD; these B cells are believed to play numerous pathogenic roles such as secreting autoantibodies, secreting pro-inflammatory cytokines or even activating a cell-mediated response by serving as antigen processing cells. The pathogenicity of these antibodies makes it imperative to identify their target antigens, however the increased reactivity to self-antigens as well as phenomenon like epitope spreading add to the complexity of identifying a 'trigger antigen'. Numerous hypotheses attempt to explain this influx of autoantigens in autoimmunity, from molecular mimicry by pathogens to release of intracellular antigens due to frequent cell death.

In terms of IgG4-RD, very little is known. Such autoantigens have been described in relevant conditions like type 1 autoimmune pancreatitis (AIP), where patients were found to express putative autoantibodies against carbonic anhydrases¹⁰, lactoferrin¹¹, and several pancreatic and salivary antigens¹². However, these autoantigens have not been studied in the context of IgG4-RD; identifying such antigens and their correlation to organ manifestations can

provide great insight into the pathogenesis underlying IgG4-RD. Importantly, it can also provide possible therapeutic targets to halt the robust autoimmune response in patients.

Our experimental findings revealed some interesting correlations. Firstly, we illustrated that the autoantigens previously identified in AIP were seen across our heterogenous IgG4-RD cohort (Figure 21). In fact, only 7 of our 33 patients positive for autoantibodies were AIP, thus these autoantigens are not limited to AIP but also present in other organ manifestations in IgG4-RD. (Figure 22).

This led us to question if reactivity to autoantigens correlated with a patient's organ involvement. To analyze this statistically, we used a Fischers exact test and were surprised to see a significant correlation between involvement of lungs, other ENT and sclerosing mediastinitis and whether patients had autoreactive antibodies. This was noteworthy, especially since 4 out of the 6 patients positive for PRSS2 had lung manifestations. It may be interesting to elucidate the pathogenesis in these patients and if it differs from other IgG4-RD patients, i.e. are they more prone to a loss of tolerance?

Another key finding in our project was the presence of multiple IgG subclasses specific to each autoantigen. This seemed striking since it alluded to the idea of interplay between IgG subclasses and highlighted the complexity of the immune response. It further raises questions such as why are IgG1 and IgG4 predominantly expressed? Are their functions synchronous or antagonistic? Analyzing the differences in IgG subclasses in context of different autoantigens may provide us with a better understanding of the role of B cells in IgG4-RD.

Future Directions

Due to the limitations discussed in the results sections, we are incorporating a highly sensitive, antibody-profiling assay— Luciferase Immunoprecipitation System (LIPS)^{8, 13}. This will allow us to test patient antibody specificity against the 20 PLATO proteins.

In addition, we will simultaneously be synthesizing recombinant monoclonal immunoglobulins (heavy and light chains) from patients with active IgG4-RD. These immunoglobulins will then be exposed to whole cell lysate and their antigenic targets will be identified.

Finally, we will confirm if the antigen is sufficient to cause T-cell proliferation, using a cytotoxic T cell proliferation assay containing CD4+ cytotoxic T lymphocytes, antigen, and peripheral blood mononuclear cells.

Conclusion

Our results identified twenty potential antigenic targets in IgG4-RD and studied autoantigens that were seen in several autoimmune disorders but had not been analyzed in the context of IgG4-RD. Our results, namely the presence of autoantigen specific antibodies, suggest that there may indeed be a breach in tolerance against self-antigens, consequently producing autoantibodies which mediate pathogenic effector functions and instigation of clinical symptoms¹⁴. However, our results brought up numerous questions regarding heterogeneity between patients, in terms of autoantigen reactivity as well as autoreactivity of varying IgG

subclasses. I hope to be answer these questions through future experiments and gain insight into the pathogenic mechanism in IgG4-RD.

Although it has been a little over a decade since the recognition of IgG4-RD, we have learnt an immense amount about the disease, from an accurate diagnosis to treating with Rituximab. Nevertheless, determining the primary autoantigen remains a crucial step in unfolding the pathogenesis underlying IgG4-RD. Gaining a through understanding of disease etiology may currently seem like an enormous challenge, but it is one that we must overcome to develop new and effective therapeutics for patients.

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